METHODS AND DEVICES FOR OBTAINING NON-HEMATOPOIETIC LINEAGE CELLS FROM HEMATOPOIETIC PROGENITOR CELLS

Field of the Invention

This invention relates generally to hematopoietic cells, and more specifically to methods and devices for obtaining non-hematopoietic lineage cells from hematopoietic progenitor cells. Non-hematopoietic lineage cells obtained by the foregoing methods of the invention are useful in the therapeutic treatment of a variety of pathologic conditions in humans and other species.

Background of the Invention

The hematopoietic system is formed via a complex series of event at the molecular and cellular levels in which pluripotent hematopoietic stem cells give rise to mature, functional daughter cells. Hematopoietic stem cells begin blood cell formation early in embryogenesis, going through a series of anatomic moves that take them from the yolk sac to the liver and spleen and ultimately to the bone marrow, where they reside from the neonate stage through adulthood. In the bone marrow, the hematopoietic cells interact with a variety of other cell types, including stromal/mesenchymal cells, endothelial cells, fat cells and bone cells, among others, to provide appropriate hematopoietic homeostasis. Through extensive investigations over the past few decades, discrete lineage progression pathways have been described that define the successive differentiation steps of immature hematopoietic stem cells into a variety of peripheral blood cells, including red blood cells, platelets, granulocytes, monocytes/macrophages, lymphocytes, and antigen presenting cells. This work has demonstrated that as the source stem cells divide and mature into daughter progeny, they become progressively more 'committed' to the ultimate cell type toward which they are directed, and lose their multipotent ability to form the full array of hematopoietic cells.

In undertaking the sequential maturation steps typical of hematopoiesis, hematopoietic cells undergo a series of phenotypic changes that broadly correlate with functional capabilities. Traditionally, the most immature hematopoietic progenitor (or stem) cells have been characterized as expressing a cell surface molecule termed $CD34^+$. As the cells mature through proliferative and differentiation stages, the expression of CD34 on the cell surface is lost, while other, lineage-specific antigens are acquired. Interestingly, it has been reported that the most immature hematopoietic cells may not express CD34 either (in conjunction with their lack of lineage specific markers). Thus, hematopoietic progenitor cells (HPCs) appear

to progress from $CD34^-lin^-$ stage, to $CD34^+$ lin^-, to $CD34^+$ lin^+, and finally to $CD34^-lin^+$. Hematopoietic progenitor cells ($CD34^-lin^-$ and/or $CD34^+$ lin^-), are typically envisioned to reside in the bone marrow, although they can be isolated from umbilical cord and placental blood, from fetal liver and other fetal tissues such as the yolk sac, and from peripheral blood at low frequencies which can be increased by the administration of certain chemotherapeutic drugs.

While the pluripotent characteristics of HPCs have been reported to enable the formation of various mature blood cells, the capabilities of these cells in forming other, non-hematopoietic tissues has not been defined. Recent reports have generated intriguing data on the relative 'stem like' attributes of various hematopoietic cells. For example, there have been anecdotal reports of the possible ability of HSCs to form muscle cells, but these reports have been confounded by the possibility that the derived muscle cells may have arisen from contaminating muscle progenitor cells (Ferrari et al., 1998, Bittner et al., 1999).

A successful approach providing a common progenitor cell would greatly facilitate the production of a large number of further differentiated precursor cells of a specific lineage including non-hematopoietic cells, and in turn provide a larger number of differentiated cells with a wide variety of applications, including blood transfusions and cell-lineage specific cell reconstitutions including tissue regeneration and organ replacement.

An object of the invention is to provide methods and devices for the controlled production in large numbers of specific lineages of progenitor cells and their more differentiated cells.

Summary of the Invention

The invention, in one important part, involves methods and devices for obtaining non-hematopoietic lineage cells from hematopoietic progenitor cells. Thus, one aspect of the invention is the variety of differentiated non-blood progeny cells and tissue, including in forms suitable for medical use, that can be obtained from a sample of hematopoietic progenitor cells.

Surprisingly, according to the invention, it has been discovered that under appropriate conditions, different fractions of hematopoietic progenitor cells are capable of forming an array of cell and tissue types, including mesenchymal, parenchymal, neuronal, endothelial, and epithelial cells. The invention thus involves this unexpected tissue multipotency of hematopoietic progenitor cells.

We have also discovered, unexpectedly, that such differentiation can be carried out with greater efficiency in a three dimensional matrix culture device as compared to standard two-dimensional (or monolayer) culture devices.

The invention has therefore a variety of therapeutic applications in tissue repair, tissue transplantation, tissue re-implantation, tissue-specific expression of recombinant genes, and the like. Examples of such tissues include, but are not limited to, brain tissue, breast tissue, gastrointestinal tissue, ovarian tissue, and/or tissue of the following organs and/or systems: Blood and Blood Forming system: including platelets, blood vessel wall, and bone marrow; Cardiovascular system: including heart and vascular system; Digestive and excretory system: including alimentary tract, biliary tract, kidney, liver, pancreas and urinary tract; Endocrine system: including adrenal gland, kidney, ovary, pituitary gland, renal gland, salivary gland, sebaceous gland, testis, thymus gland and thyroid gland; Muscular system: including muscles that move the body; Reproductive System: including breast, ovary, penis and uterus; Respiratory system: including bronchus, lung and trachea; Skeletal system: including bones and joints; Tissue, fiber, and integumentary system: including adipose tissue, cartilage, connective tissue, cuticle, dermis, epidermis, epithelium, fascia, hair follicle, ligament, bone marrow, melanin, melanocyte, mucous membrane, skin, soft tissue, synovial capsule and tendon.

According to one aspect of the invention, a method for *in vitro* culture of hematopoietic progenitor cells to produce differentiated cells of non-hematopoietic lineage, is provided. The method involves culturing hematopoietic progenitor cells in an environment that promotes hematopoietic progenitor cell differentiation, under conditions and for a period of time to produce differentiated cells of non-hematopoietic lineage.

In some embodiments, the environment comprises factors that direct differentiation of hematopoietic progenitor cells to produce differentiated cells of non-hematopoietic lineage selected from the group consisting of mesenchymal, parenchymal, neuronal, endothelial, and epithelial cells. In a certain embodiment, the hematopoietic progenitor cells are $CD34^-$ cells, and the environment comprises growth factors selected from the group consisting of bFGF and TGF-β, to produce mesenchymal cells. In a further embodiment, the hematopoietic progenitor cells are $CD34^+$ and/or $CD34^-$ cells, and the environment comprises growth factors selected from the group consisting of putrescine, progesterone, sodium selenite, insulin, transferrin, EGF, NGF, and bFGF, to produce neuronal cels. In a yet further embodiment, the hematopoietic progenitor cells are $CD34^+$ and/or $CD34^-$, and the environment comprises

growth factors selected from the group consisting of IL-3, SCF, TGF-β1, and Flk-2/Flt-3 ligand, to produce epithelial cells. In a yet further embodiment, the hematopoietic progenitor cells are $CD34^+$ and/or $CD34^-$, and the environment comprises VEGF, to produce endothelial cells. In a still further embodiment the hematopoietic progenitor cells are $CD34^+$ and/or $CD34^-$, and the environment comprises EGF, bFGF, and SF/HGF, to produce parenchymal cells.

In important embodiments, the environment comprises a solid, porous matrix having a unitary microstructure. In certain embodiments, the environment comprises factors that direct differentiation of hematopoietic progenitor cells to produce differentiated cells of non-hematopoietic lineage selected from the group consisting of mesenchymal, parenchymal, neuronal, endothelial, and epithelial cells.

In a further embodiment of the invention, the cells are cultured upon and within a solid, porous matrix. The porous matrix can be one that is an open cell porous matrix having a percent open space of at least 50%, and preferably at least 75%. In one embodiment the porous solid matrix has pores defined by interconnecting ligaments having a diameter at midpoint, on average, of less than 150 μm. Preferably the porous solid matrix is a metal-coated reticulated open cell foam of carbon containing material, the metal coating being selected from the group consisting of tantalum, titanium, platinum (including other metals of the platinum group), niobium, hafnium, tungsten, and combinations thereof. In preferred embodiments, whether the porous solid matrix is metal-coated or not, the matrix is coated with a biological agent selected from the group consisting of collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors, glycosaminoglycans, vitrogen, antibodies and fragments thereof, functional equivalents of these factors, and combinations thereof. Most preferably the metal coating is tantalum coated with a biological agent. In certain other embodiments the porous solid matrix having seeded hematopoietic progenitor cells and their progeny is impregnated with a gelatinous agent that occupies pores of the matrix. The preferred embodiments of the invention are solid, unitary macrostructures, i.e. not beads or packed beads. They also involve nonbiodegradable materials.

In other embodiments, the hematopoietic progenitor cells are obtained from a blood product. Preferably the blood product is unfractionated bone marrow.

As will be understood, according to the invention, it is possible now to culture hematopoietic progenitor cells and to harvest non-hematopoietic progenitor cells.

In one embodiment, the hematopoietic progenitor cells are enriched for the $CD34^-$ cells. In another embodiment, the hematopoietic progenitor cells are enriched for the $CD34^+$ cells. In another embodiment, the method further involves first isolating the hematopoietic cells from nonnucleated cells. In still another embodiment, the method involves enriching the hematopoietic progenitor cells for a subpopulation of hematopoietic progenitor cells having a specific marker for a specific tissue, as described in greater detail below.

According to another aspect of the invention, a method is provided for producing a selected nonhematopoietic tissue from hematopoietic progenitor cells. The method involves the *in vitro* culture of cells as described in detail above.

According to yet another aspect of the invention, a method is provided for treating a subject. The method involves culturing hematopoietic progenitor cells as described above to produce nonhematopoietic cells and/or tissue, and then administering the nonhematopoietic cells and/or implanting the tissue into the subject. The treatment may be administration of cells, and/or transplantation or reimplantation of cells or tissue. In important embodiments, hematopoietic progenitor cells may be genetically altered. In further important embodiments, nonhematopoietic cells are administered into a subject intravenously. The treatment may be for treating any number of disorders, all of which are described in greater detail below. The various conditions of culture are as described above, as if fully repeated in this paragraph.

These and other aspects of the invention, as well as various advantages and utilities, will be more apparent with reference to the detailed description of the preferred embodiments.

Brief Description of the Drawings

Figure 1. Graph of relative cell yields of hematopoietic cell-derived vimentin positive epithelial cells in three dimensional Cellfoam matrix cultures compared to plastic monolayer control cultures.

Figure 2. Graph of relative yields of hematopoietic cell-derived neurofilament positive neuronal cells in three dimensional Cellfoam matrix cultures compared to plastic monolayer control cultures.

Figure 3. Flow cytometric analysis charts of $CD34^-Lin^-$ cells cultured for 4 weeks in three different cell growth media; Fig. 3A shows the presence of myeloid $CD45^+CD33^+$ cells in hematopoietic cell growth media of the $CD34^-Lin^-$ cultures; Fig. 3B shows the presence of endothelial cells (left panel), and of epithelial cells (right panel) in the mesenchymal cell growth cultures of $CD34^-Lin^-$ cells; Fig. 3C shows the presence of neurofilament positive

cells in the $CD34^-Lin^-$ cells cultured neural cell growth media (Left panel: isotype control; right panel: cultured cells).

Figure 4. Flow cytometric analysis charts of brain cells from control (left panel), and transplanted mice with human $CD34^-Lin^-$ cells (right panel), showing the detection of human cell surface antigen expression (CD45).

Figure 5. PCR amplification results showing the presence or absence of human globin DNA in the brain and muscle transplanted and control mice.

Brief Description of the Sequences

SEQ ID NO:1 is a human-specific globin PCR primer.

SEQ ID NO:2 is a human-specific globin PCR primer.

SEQ ID NO:3 is a human-specific globin PCR primer.

Detailed Description of the Invention

The invention, in one important part, involves methods and devices for obtaining non-hematopoietic lineage cells from hematopoietic progenitor cells. Thus, one aspect of the invention is the variety of differentiated non-blood progeny cells that can be obtained from a sample of hematopoietic progenitor cells.

Surprisingly, according to the invention, it has been discovered that under appropriate conditions, different fractions of hematopoietic progenitor cells are capable of forming an array of cell and tissue types, including mesenchymal, parenchymal, neuronal, endothelial, and epithelial cells. The invention thus involves this unexpected tissue multipotency of hematopoietic progenitor cells.

We have also discovered, unexpectedly, that such differentiation can be carried out with greater efficiency in a three dimensional matrix culture device as compared to standard two-dimensional (or monolayer) culture devices.

The invention, in one important embodiment, involves culturing hematopoietic progenitor cells in a porous solid matrix. A porous, solid matrix, is defined as a three-dimensional structure with "sponge-like" continuous pores forming an interconnecting network. The matrix can be rigid or elastic, and it provides a scaffold upon which cells can grow throughout. Its pores are interconnected and provide the continuous network of channels extending through the matrix and also permit the flow of nutrients throughout. A preferred matrix is an open cell foam matrix having a percent open space of at least 50% and preferably 75%. Thus, it is preferred that the open space comprise the majority of the matrix.

This is believed to maximize cell migration, cell-cell contact, space for cell growth and accessibility to nutrients. It is preferred that the porous matrix be formed of a reticulated matrix of ligaments which at their center point are less than 150 μm in diameter, preferably 60 μm, whereby a cell can reside on or interact with a portion of the ligament. Preferably, the average pore diameter is on the order of 300 μm, which resembles cancellous bone. Suitable matrices can be obtained using a number of different methods. Examples of such methods include solvent casting or extraction of polymers, track etching of a variety of materials, foaming of a polymer, the replamineform process for hydroxyapatite, and other methodologies well known to those of ordinary skill in the art. The materials employed can be natural or synthetic, including biological materials such as proteins, hyaluronic acids, synthetic polymers such as polyvinyl pyrolidones, polymethylmethacrylate, methyl cellulose, polystyrene, polypropylene, polyurethane, ceramics such as tricalcium phosphate, calcium aluminate, calcium hydroxyapatite and ceramic-reinforced or coated polymers. If the starting material for the scaffold is not metal, a metal coating can be applied to the three-dimensional matrix. Metal coatings provide further structural support and/or cell growth and adhesive properties to the matrix. Preferred metals used as coatings comprise tantalum, titanium, platinum and metals in the same element group as platinum, niobium, hafnium, tungsten, and combinations of alloys thereof. Coating methods for metals include a process such as CVD (Chemical Vapor Deposition). The preferred matrix, refered to herein throughout as Cellfoam, is described in detail in U.S. Patent No. 5,282,861, and is incorporated herein by reference ("Cellfoam", Cytomatrix, Woburn, MA). More specifically, the preferred matrix is a reticulated open cell substrate formed by a lightweight, substantially rigid foam of carbon-containing material having open spaces defined by an interconnecting network, wherein said foam material has interconnected continuous channels, and a thin film of metallic material deposited onto the reticulated open cell substrate and covering substantially all of the interconnecting network to form a composite porous biocompatible material creating a porous microstructure similar to that of natural cancellous bone.

Additionally, such matrices can be coated with biological agents which can promote cell adhesion for the cultured hematopoietic cells, allowing for improved migration, growth and proliferation. Moreover, when these matrices are used for the *in vivo* maintenance, expansion and/or differentiation of hematopoietic progenitor cells (i.e., when the matrices with the cells are implanted into a subject, -see also discussion below), biological agents that promote angiogenesis (vascularization) and biological agents that prevent/reduce

inflammation may also be used for coating of the matrices. Preferred biological agents comprise collagens, fibronectins, laminins, integrins, angiogenic factors, anti-inflammatory factors, glycosaminoglycans, vitrogen, antibodies and fragments thereof, functional equivalents of these agents, and combinations thereof.

Angiogenic factors include platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), bFGF-2, leptins, plasminogen activators (tPA, uPA), angiopoietins, lipoprotein A, transforming growth factor-β, bradykinin, angiogenic oligosaccharides (e.g., hyaluronan, heparan sulphate), thrombospondin, hepatocyte growth factor (also known as scatter factor) and members of the CXC chemokine receptor family. Anti-inflammatory factors comprise steroidal and non-steroidal compounds and examples include: Alclofenac; Alclometasone Dipropionate; Algestone Acetonide; Alpha Amylase; Amcinafal; Amcinafide; Amfenac Sodium; Amiprilose Hydrochloride; Anakinra; Anirolac; Anitrazafen; Apazone; Balsalazide Disodium; Bendazac; Benoxaprofen; Benzydamine Hydrochloride; Bromelains; Broperamole; Budesonide; Carprofen; Cicloprofen; Cintazone; Cliprofen; Clobetasol Propionate; Clobetasone Butyrate; Clopirac; Cloticasone Propionate; Cormethasone Acetate; Cortodoxone; Deflazacort; Desonide; Desoximetasone; Dexamethasone Dipropionate; Diclofenac Potassium; Diclofenac Sodium; Diflorasone Diacetate; Diflumidone Sodium; Diflunisal; Difluprednate; Diftalone; Dimethyl Sulfoxide; Drocinonide; Endrysone; Enlimomab; Enolicam Sodium; Epirizole; Etodolac; Etofenamate; Felbinac; Fenamole; Fenbufen; Fenclofenac; Fenclorac; Fendosal; Fenpipalone; Fentiazac; Flazalone; Fluazacort; Flufenamic Acid; Flumizole; Flunisolide Acetate; Flunixin; Flunixin Meglumine; Fluocortin Butyl; Fluorometholone Acetate; Fluquazone; Flurbiprofen; Fluretofen; Fluticasone Propionate; Furaprofen; Furobufen; Halcinonide; Halobetasol Propionate; Halopredone Acetate; Ibufenac; Ibuprofen; Ibuprofen Aluminum; Ibuprofen Piconol; Ilonidap; Indomethacin; Indomethacin Sodium; Indoprofen; Indoxole; Intrazole; Isoflupredone Acetate; Isoxepac; Isoxicam; Ketoprofen; Lofemizole Hydrochloride; Lornoxicam; Loteprednol Etabonate; Meclofenamate Sodium; Meclofenamic Acid; Meclorisone Dibutyrate; Mefenamic Acid; Mesalamine; Meseclazone; Methylprednisolone Suleptanate; Morniflumate; Nabumetone; Naproxen; Naproxen Sodium; Naproxol; Nimazone; Olsalazine Sodium; Orgotein; Orpanoxin; Oxaprozin; Oxyphenbutazone; Paranyline Hydrochloride; Pentosan Polysulfate Sodium; Phenbutazone Sodium Glycerate; Pirfenidone; Piroxicam; Piroxicam Cinnamate; Piroxicam Olamine; Pirprofen; Prednazate; Prifelone; Prodolic Acid;

Proquazone; Proxazole; Proxazole Citrate ; Rimexolone; Romazarit ; Salcolex ; Salnacedin; Salsalate ; Sanguinarium Chloride ; Seclazone ; Sermetacin; Sudoxicam; Sulindac; Suprofen; Talmetacin; Talniflumate; Talosalate; Tebufelone; Tenidap; Tenidap Sodium; Tenoxicam; Tesicam; Tesimide; Tetrydamine ; Tiopinac ; Tixocortol Pivalate; Tolmetin; Tolmetin Sodium; Triclonide; Triflumidate; Zidometacin; Zomepirac Sodium.

The cells cultured according to the methods of the invention are hematopoietic progenitor cells. "Hematopoietic progenitor cells" as used herein refer to immature blood cells having the capacity to self-renew and to differentiate into the more mature blood cells (also described herein as "progeny") comprising granulocytes (e.g., promyelocytes, neutrophils, eosinophils, basophils), erythrocytes (e.g., reticulocytes, erythrocytes), thrombocytes (e.g., megakaryoblasts, platelet producing megakaryocytes, platelets), and monocytes (e.g., monocytes, macrophages). Such cells, according to the present invention, may include $CD34^+$ cells and/or $CD34^-$ cells.

"Mature blood cells" (or "progeny"), as used herein, refer to cells that express one or more cell surface antigen(s) characteristic of a hematopoietic lineage cell. Such cell surface antigen(s) are well known to those of ordinary skill in the art, and include CD33, CD19, CD61, CD71, CD42, CD66, CD66e, CD3, CD4, CD8, CD2, and CD20.

$CD34^+$ cells are immature cells present in the "blood products" described below, express the CD34 cell surface marker, and are believed to include a subpopulation of cells with the "progenitor cell" properties defined above. The likewise immature $CD34^-$ subset of bone marrow cells also possesses important progenitor cell capabilities, including the ability to reconstitute the hematopoietic system of animals. It is believed that $CD34^-$ cells, which are more fibroblast-like in nature, begin to express CD34 during hematopoietic differentiation while simultaneously self-renewing into more $CD34^-$ cells. Upon acquisition of threshold levels of CD34, which are highly charged cell surface molecules, the cell detaches from the extracellular matrix of the bone marrow microenvironment and begins steps toward terminal differentiation. The $CD34^-$ precursors are highly quiescent and exhibit better engraftment capabilities than $CD34^+$ cells. As these cells mature, they acquire CD34 antigen, first at low levels and subsequently at higher levels, a progression associated with increasing maturity and proliferative ability. It has also been reported that $CD34^-$ cells are unresponsive to cytokines in traditional methylcellulose assays and do not form colonies.

The hematopoietic progenitor cells can be obtained from blood products. A "blood product" as used in the present invention defines a product obtained from the body or an organ of the body containing cells of hematopoietic origin. Such sources include

unfractionated bone marrow, umbilical cord, peripheral blood, liver, thymus, lymph and spleen. It will be apparent to those of ordinary skill in the art that all of the aforementioned crude or unfractionated blood products can be enriched for cells having "hematopoietic progenitor cell" characteristics in a number of ways. For example, the blood product can be depleted from the more differentiated progeny. The more mature, differentiated cells can be selected against, via cell surface molecules they express. Additionally, the blood product can be fractionated selecting for $CD34^+$ cells. As mentioned earlier, $CD34^+$ cells are thought in the art to include a subpopulation of cells capable of self-renewal and pluripotentiality. Such selection can be accomplished using, for example, commercially available magnetic anti-CD34 beads (Dynal, Lake Success, NY). Unfractionated blood products can be obtained directly from a donor or retrieved from cryopreservative storage.

Employing the culture conditions described in greater detail below, it is possible according to the invention to preserve hematopoietic progenitor cells and to stimulate the expansion of hematopoietic progenitor cell number and/or colony forming unit potential. Once expanded, the cells, for example, can be differentiated into nonhematopoietic tissue and returned to the body to supplement, replenish, etc. a patient's nonhematopoietic progenitor cell population. The invention thus is useful for therapeutic purposes and also is useful for research purposes such as in testing in animal or *in vitro* models of medical, physiological or metabolic pathways or conditions, and/or in the *in vitro* studying of cell differentiation.

It also is possible to take the increased numbers of hematopoietic progenitor cells produced according to the invention and stimulate them with hematopoietic growth agents that promote hematopoietic cell maintenance, expansion and/or differentiation, to yield the more mature blood and non-blood cells, *in vitro*. Such expanded populations of blood and non-blood cells may be applied *in vivo* as described above, or may be used experimentally as will be recognized by those of ordinary skill in the art. Such differentiated cells include those described above, and elsewhere herein.

In the preferred embodiments of the invention, the hematopoietic progenitor cells are continuously cultured for an extended period of time, and aliquots of the cultured cells are harvested spaced apart in time or intermittently. "Harvesting hematopoietic cells" is defined as the dislodging or separation of cells from the matrix. This can be accomplished using a number of methods, such as enzymatic, centrifugal, electrical or by size, or the one preferred in the present invention, by flushing of the cells using the media in which the cells are incubated. The cells can be further collected and separated. "Harvesting steps spaced apart in time" or "intermittent harvest of cells" is meant to indicate that a portion of the cells are

harvested, leaving behind another portion of cells for their continuous culture in the established media, maintaining a continuous source of the original cells and their characteristics. Harvesting "at least a portion of" means harvesting a subpopulation of or the entirety of. Thus, as will be understood by one of ordinary skill in the art, the invention can be used to expand the number of hematopoietic progenitor cells, all the while harvesting portions of those cells being expanded for treatment to develop even larger populations of differentiated cells.

In all of the culturing methods according to the invention, except as otherwise provided, the media used is that which is conventional for culturing cells. Examples include RPMI, DMEM, ISCOVES, etc. Typically these media are supplemented with human or animal plasma or serum. Such plasma or serum can contain small amounts of hematopoietic growth factors. The media used according to the present invention, however, can depart from that used conventionally in the prior art. In particular, it has been discovered, that hematopoietic progenitor cells can be cultured on the matrices described above for extended periods of time without the need for adding any exogenous growth agents (other than those which may be contained in plasma or serum, hereinafter "serum"), without inoculating the environment of the culture with stromal cells and without using stromal cell conditioned media. The growth agents of particular interest in connection with the present invention are growth factors. "Growth factors," as used herein, refer to factors that influence the survival, proliferation or differentiation of hematopoietic cells to blood or non-blood cells. Growth factors that promote differentiation of hematopoietic progenitor toward a non-blood cell lineage include, but are not limited to bFGF, TGF-β, TGF-β1, putrescine, progesterone, sodium selenite, insulin, transferrin, EGF, NGF, IL-3, SCF, and Flk-2/Flt-3 ligand, VEGF, SF/HGF and the like. The foregoing factors are well known to those of ordinary skill in the art. Most are commercially available. They can be obtained by purification, by recombinant methodologies or can be derived or synthesized synthetically.

In one aspect of the invention, the hematopoietic progenitor cells are cultured in an environment that may include inoculated stromal cells, stromal cell conditioned medium and growth factors that promote and direct differentiation of hematopoietic cells toward a specific non-hematopoietic cell-lineage. By "inoculated" stromal cells, it is meant that the cell culture includes stromal cells which have been introduced into the chamber as an inoculum for promoting survival, proliferation, and/or lineage-specific differentiation of the hematopoietic progenitor cells.

"Stromal cells" as used herein comprise fibroblasts and mesenchymal cells, with or without other cells and elements, and can be seeded prior to, or substantially at the same time as, the hematopoietic progenitor cells, therefore establishing conditions that favor the subsequent attachment and growth of hematopoietic progenitor cells. Fibroblasts can be obtained via a biopsy from any tissue or organ, and include fetal fibroblasts. These fibroblasts and mesenchymal cells may be transfected with exogenous DNA that encodes, for example, one of the hematopoietic growth factors described above.

"Stromal cell conditioned medium" refers to medium in which the aforementioned stromal cells have been incubated. The incubation is performed for a period sufficient to allow the stromal cells to secrete factors into the medium. Such "stromal cell conditioned medium" can then be used to supplement the culture of hematopoietic progenitor cells promoting their proliferation and/or lineage-specific differentiation according to the invention.

All of the foregoing stromal cells may be genetically manipulated so that they express a growth factor according to the invention, for example, a factor that directs hematopoietic progenitor cells toward a specific, hematopoietic and/or non-hematopoietic lineage.

The culture of the hematopoietic cells preferably occurs under conditions sufficient to increase the number of such cells, the colony forming potential of such cells, and/or the differentiation of such cells into hematopoietic and non-hematopoietic cell lineages. The conditions used refer to a combination of conditions known in the art (e.g., temperature, CO_2 and O_2 content, nutritive media, etc.). The time sufficient to increase the number of cells is a time that can be easily determined by a person skilled in the art, and can vary depending upon the original number of cells seeded. As an example, discoloration of the media can be used as an indicator of confluency. Additionally, and more precisely, different volumes of the blood product can be cultured under identical conditions, and cells can be harvested and counted over regular time intervals, thus generating the "control curves". These "control curves" can be used to estimate cell numbers in subsequent occasions.

The conditions for determining colony forming potential are similarly determined. Colony forming potential is the ability of a cell to form progeny. Assays for this are well known to those of ordinary skill in the art and include seeding cells into a semi-solid, treating them with growth factors and counting the number of colonies.

According to another aspect of the invention a method for *in vivo* therapeutic application of cells produced according to the methods of the present invention is provided. In one embodiment, the method involves implanting cells produced according to the methods

of the present invention into a subject. In a further embodiment, the method involves implanting a porous solid matrix having pre-seeded hematopoietic progenitor cells and hematopoietic progenitor cell progeny. Implantation of matrices similar to the matrices of the invention is well known in the art (Stackpool, GJ, et al, Combined Orthopaedic Research Societies Meeting, Nov. 6-8, 1995, San Diego, CA, Abstract Book p. 45; Turner, TM, et al., 21st Annual Meeting of the Society for Biomaterials, March 18-22, San Francisco, CA, Abstract Book p. 125). Such matrices are biocompatible (i.e., no immune reactivity-no rejection) and can be implanted and transplanted in a number of different tissues of a subject. Such methods are useful in a variety of ways, including the study of hematopoietic progenitor cell maintenance, expansion and/or differentiation *in vivo*, in a number of different tissues of a subject, or in different subjects.

As used herein, a subject is a human, non-human primate, cow, horse, pig, sheep, goat, dog, cat or rodent. Human hematopoietic progenitor cells and human subjects are particularly important embodiments. As descibed above, when the matrices of the invention are used for such *in vivo* implantation studies, biological agents that promote angiogenesis (vascularization) and/or prevent/reduce inflammation may also be used for coating of the matrices. Preferred biological agents are as described above. Also as described above, the hematopoietic progenitor cells are pre-seeded onto the porous solid matrix and cultured *in vitro* according to the invention, before implantation into a subject. According to the invention, an amount of the cells is introduced *in vitro* into the porous solid matrix, and cultured in an environment that may contain inoculated stromal cells, stromal cell conditioned medium, and exogenously added hematopoietic growth factors that promote hematopoietic cell maintenance, expansion and/or differentiation, other than serum. Implantation is then carried out.

In certain embodiments of the invention the porous solid matrix having seeded hematopoietic progenitor cells, without or preferably with their differentiated non-blood cell progeny, is impregnated with a gelatinous agent that occupies pores of the matrix. By "seeded" it is meant that the hematopoietic progenitor cells, with or without their progeny, are seeded prior to, substantially at the same time as, or following impregnation (or infiltration) with a gelatinous agent. For example, the cells may be mixed with the gelatinous agent and seeded at the same time as the impregnation of the matrix with the agent. In some embodiments, the hematopoietic progenitor cells, with or without their progeny, are pre-seeded onto the porous solid matrix. According to the invention, an amount of the cells is introduced *in vitro* into the porous solid matrix, and cultured in an environment that is free of

inoculated stromal cells, stromal cell conditioned medium, and exogenously added hematopoietic growth factors that promote hematopoietic cell maintenance, expansion and/or differentiation, other than serum.

"Impregnation" with a gelatinous agent serves as to contain the cells within the matrix, and also to help maintain and/or enhance cell attachment onto the matrix. The "gelatinous" agent may be one that can be maintained in a fluid state initially, and after its application into the matrix, be gelatinized *in situ* in the matrix. Such gelatinization may occur in a number of different ways, including altering the agent's temperature, irradiating the agent with an energy source (e.g., light), etc. The agent may exist in a continuum from a fluid state to a semi-solid (gelatinous) state to a solid state. An agent's final state and gelatinization will always depend upon the particular "gelatinous" agent used and its individual properties. A preferred "gelatinous" agent is characterized also by its structural porosity, necessary for allowing the nutrients of the growth media to reach the cells throughout the matrix. Exemplary "gelatinous" agents include cellulosic polysaccharides (such as cellulose, hemicellulose, methylcellulose, and the like), agar, agarose, albumin, algal mucin, mucin, mucilage, collagens, glycosaminoglycans, and proteoglycans (including their sulphated forms). In certain embodiments, the gelatinous agent may impregnate the matrix completely, in some embodiments partially, and in other embodiments minimally, serving only as a coating of the outer surfaces of the matrix. The extent of the impregnation will largely depend upon the physical characteristics of the "gelatinous" agent of choice. In preferred embodiments the "gelatinous" agent is methylcellulose and the impregnation is complete.

The invention also involves the unexpected discovery that differentiated cells of non-hematopoietic lineage produced from hematopoietic progenitor cells according to the methods of the invention, when administered intravenously to a subject they specifically "home-in" to a tissue comprising cells of a lineage similar to the lineage of the administered differentiated cell. For example, cells produced from hematopoietic progenitor cells (e.g., from $CD34^{-}Lin^{-}$ cells) under neuronal growth conditions, when injected intravenously into a subject "home-in" to the neuronal tissue of the subject (e.g., brain) (see also under the Examples section). The invention is therefore particularly useful for the delivery of cells of a specific lineage to a tissue normally comprising such cells, and/or for the tissue-specific delivery of exogenous nucleic acids and polypeptides.

For example, if the tissue-specific delivery of a polypeptide is needed (e.g., to replenish a missing polypeptide, or to provide new, desirable attributes to a tissue), hematopoietic progenitor cells could be genetically altered (e.g., transduced) to express the

polypeptide of choice, differentiated to a specific nonhematopoietic lineage according to any of the foregoing methods of the invention, and then administered to a subject in need of such treatment according to any acceptable methods of administration discussed elsewhere herein.

According to the present invention, hematopoietic progenitor cells can be more efficiently transduced if the transduction occurs while the hematopoietic progenitor cells are on and within a solid porous matrix as described above. When the hematopoietic progenitor cells are differentiated according to the invention, the specific-lineage progeny would therefore express the transduced gene of interest. As used herein, "transduction of hematopoietic cells" refers to the process of transferring exogenous genetic material into a cell of hematopoietic origin. The terms "transduction", "transfection" and "transformation" are used interchangeably throughout this letter, and refer to the process of transferring exogenous genetic material into a cell (i.e., to produce genetically altered cells). As used herein, "exogenous genetic material" refers to nucleic acids or oligonucleotides, either natural or synthetic, that are introduced into the hematopoietic progenitor cells. The exogenous genetic material may be a copy of that which is naturally present in the cells, or it may not be naturally found in the cells. It typically is at least a portion of a naturally occuring gene which has been placed under operable control of a promoter in a vector construct.

Various techniques may be employed for introducing nucleic acids into cells. Such techniques include transfection of nucleic acid-$CaPO_4$ precipitates, transfection of nucleic acids associated with DEAE, transfection with a retrovirus including the nucleic acid of interest, liposome mediated transfection, and the like. For certain uses, it is preferred to target the nucleic acid to particular cells. In such instances, a vehicle used for delivering a nucleic acid according to the invention into a cell (e.g., a retrovirus, or other virus; a liposome) can have a targeting molecule attached thereto. For example, a molecule such as an antibody specific for a surface membrane protein on the target cell or a ligand for a receptor on the target cell can be bound to or incorporated within the nucleic acid delivery vehicle. For example, where liposomes are employed to deliver the nucleic acids of the invention, proteins which bind to a surface membrane protein associated with endocytosis may be incorporated into the liposome formulation for targeting and/or to facilitate uptake. Such proteins include proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half life, and the like. Polymeric delivery systems also have been used successfully to deliver nucleic acids into cells, as is known by those skilled in the art. Such systems even permit oral delivery of nucleic acids.

In the present invention, the preferred method of introducing exogenous genetic material into hematopoietic cells is by transducing the cells *in situ* on the matrix using replication- deficient retroviruses. Replication-deficient retroviruses are capable of directing synthesis of all virion proteins, but are incapable of making infectious particles. Accordingly, these genetically altered retroviral vectors have general utility for high-efficiency transduction of genes in cultured cells, and specific utility for use in the method of the present invention. Retroviruses have been used extensively for transferring genetic material into cells. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with the viral particles) are provided in the art.

The major advantage of using retroviruses is that the viruses insert efficiently a single copy of the gene encoding the therapeutic agent into the host cell genome, thereby permitting the exogenous genetic material to be passed on to the progeny of the cell when it divides. In addition, gene promoter sequences in the LTR region have been reported to enhance expression of an inserted coding sequence in a variety of cell types. The major disadvantages of using a retrovirus expression vector are (1) insertional mutagenesis, i.e., the insertion of the therapeutic gene into an undesirable position in the target cell genome which, for example, leads to unregulated cell growth and (2) the need for target cell proliferation in order for the therapeutic gene carried by the vector to be integrated into the target genome. Despite these apparent limitations, delivery of a therapeutically effective amount of a therapeutic agent via a retrovirus can be efficacious if the efficiency of transduction is high and/or the number of target cells available for transduction is high.

Yet another viral candidate useful as an expression vector for transformation of hematopoietic cells is the adenovirus, a double-stranded DNA virus. Like the retrovirus, the adenovirus genome is adaptable for use as an expression vector for gene transduction, i.e., by removing the genetic information that controls production of the virus itself. Because the adenovirus functions usually in an extrachromosomal fashion, the recombinant adenovirus does not have the theoretical problem of insertional mutagenesis. On the other hand, adenoviral transformation of a target hematopoietic cell may not result in stable transduction. However, more recently it has been reported that certain adenoviral sequences confer intrachromosomal integration specificity to carrier sequences, and thus result in a stable transduction of the exogenous genetic material.

Thus, as will be apparent to one of ordinary skill in the art, a variety of suitable vectors are available for transferring exogenous genetic material into hematopoietic cells. The selection of an appropriate vector to deliver a therapeutic agent for a particular condition amenable to gene replacement therapy and the optimization of the conditions for insertion of the selected expression vector into the cell, are within the scope of one of ordinary skill in the art without the need for undue experimentation. The promoter characteristically has a specific nucleotide sequence necessary to initiate transcription. Optionally, the exogenous genetic material further includes additional sequences (i.e., enhancers) required to obtain the desired gene transcription activity. For the purpose of this discussion an "enhancer" is simply any nontranslated DNA sequence which works contiguous with the coding sequence (in cis) to change the basal transcription level dictated by the promoter. Preferably, the exogenous genetic material is introduced into the hematopoietic cell genome immediately downstream from the promoter so that the promoter and coding sequence are operatively linked so as to permit transcription of the coding sequence. A preferred retroviral expression vector includes an exogenous promoter element to control transcription of the inserted exogenous gene. Such exogenous promoters include both constitutive and inducible promoters.

Naturally-occurring constitutive promoters control the expression of essential cell functions. As a result, a gene under the control of a constitutive promoter is expressed under all conditions of cell growth. Exemplary constitutive promoters include the promoters for the following genes which encode certain constitutive or "housekeeping" functions: hypoxanthine phosphoribosyl transferase (HPRT), dihydrofolate reductase (DHFR) (Scharfmann et al., Proc. Natl. Acad. Sci. USA 88:4626-4630 (1991)), adenosine deaminase, phosphoglycerol kinase (PGK), pyruvate kinase, phosphoglycerol mutase, the actin promoter (Lai et al., Proc. Natl. Acad. Sci. USA 86: 10006-10010 (1989)), and other constitutive promoters known to those of skill in the art. In addition, many viral promoters function constitutively in eucaryotic cells. These include: the early and late promoters of SV40; the long terminal repeats (LTRS) of Moloney Leukemia Virus and other retroviruses; and the thymidine kinase promoter of Herpes Simplex Virus, among many others. Accordingly, any of the above-referenced constitutive promoters can be used to control transcription of a heterologous gene insert.

Genes that are under the control of inducible promoters are expressed only or to a greater degree, in the presence of an inducing agent, (e.g., transcription under control of the metallothionein promoter is greatly increased in presence of certain metal ions). Inducible promoters include responsive elements (REs) which stimulate transcription when their

inducing factors are bound. For example, there are REs for serum factors, steroid hormones, retinoic acid and cyclic AMP. Promoters containing a particular RE can be chosen in order to obtain an inducible response and in some cases, the RE itself may be attached to a different promoter, thereby conferring inducibility to the recombinant gene. Thus, by selecting the appropriate promoter (constitutive versus inducible; strong versus weak), it is possible to control both the existence and level of expression of a therapeutic agent in the genetically modified hematopoietic cell. Selection and optimization of these factors for delivery of a therapeutically effective dose of a particular therapeutic agent is deemed to be within the scope of one of ordinary skill in the art without undue experimentation, taking into account the above-disclosed factors and the clinical profile of the patient.

In addition to at least one promoter and at least one heterologous nucleic acid encoding the therapeutic agent, the expression vector preferably includes a selection gene, for example, a neomycin resistance gene, for facilitating selection of hematopoietic cells that have been transfected or transduced with the expression vector. Alternatively, the hematopoietic cells are transfected with two or more expression vectors, at least one vector containing the gene(s) encoding the therapeutic agent(s), the other vector containing a selection gene. The selection of a suitable promoter, enhancer, selection gene and/or signal sequence (described below) is deemed to be within the scope of one of ordinary skill in the art without undue experimentation.

The selection and optimization of a particular expression vector for expressing a specific gene product in an isolated hematopoietic cell is accomplished by obtaining the gene, preferably with one or more appropriate control regions (e.g., promoter, insertion sequence); preparing a vector construct comprising the vector into which is inserted the gene; transfecting or transducing cultured hematopoietic cells *in vitro* with the vector construct; and determining whether the gene product is present in the cultured cells.

Table 1. Human Gene Therapy Protocols Approved by RAC: 1990-1994

Severe combined immune deficiency (SCID) due to ADA deficiency	Autologous lymphocytes transduced with human ADA gene	7/31/90
Advanced cancer	Tumor-infiltrating lymphocytes transduced with tumor necrosis factor gene	7/31/90
Advanced cancer	Immunization with autologous cancer cells transduced with tumor necrosis factor gene	10/07/91
Advanced cancer	Immunization with autologous cancer cells transduced with interleukin-2 gene	10/07/91
Asymptomatic patients infected with HIV-1	Murine Retro viral vector encoding HIV-1 genes [HIV-IT(V)]	6/07/93

AIDS	Effects of a transdominant form of *rev* gene on AIDS intervention	6/07/93
Advanced cancer	Human multiple-drug resistance (MDR) gene transfer	6/08/93
HIV infection	Autologous lymphocytes transduced with catalytic ribozyme that cleaves HIV-1 RNA (Phase I study)	9/10/93
Metastatic melanoma	Genetically engineered autologous tumor vaccines producing interleukin-2	9/10/93
HIV infection	Murine Retro viral vector encoding HIV-IT(V) genes (open label Phase I/II trial)	12/03/93
HIV infection (identical twins)	Adoptive transfer of syngeneic cytotoxic T lymphocytes (Phase I/II pilot study)	3/03/94
Breast cancer (chemo-protection during therapy)	Use of modified Retro virus to introduce chemotherapy resistance sequences into normal hematopoietic cells (pilot study)	6/09/94
Fanconi's anemia	Retro viral mediated gene transfer of the Fanconi anemia complementation group C gene to hematopoietic progenitors	6/09/94
Metastatic prostate carcinoma	Autologous human granulocyte macrophage-colony stimulating factor gene transduced prostate cancer vaccine *(first protocol to be approved under the accelerated review process; ORDA=Office of Recombinate DNA Activities)	ORDA/NIH 8/03/94*
Metastatic breast cancer	*In vivo* infection with breast-targeted Retro viral vector expressing antisense *c-fox* or antisense *c-myc* RNA	9/12/94
Metastatic breast cancer (refractory or recurrent)	Non-viral system (liposome-based) for delivering human interleukin-2 gene into autologous tumor cells (pilot study)	9/12/94
Mild Hunter syndrome	Retro viral-mediated transfer of the iduronate-2-sulfatase gene into lymphocytes	9/13/94
Advanced mesothelioma	Use of recombinant adenovirus (Phase I study)	9/13/94

The foregoing (Table 1), represent only examples of genes that can be delivered according to the methods of the invention. Suitable promoters, enhancers, vectors, etc., for such genes are published in the literature associated with the foregoing trials. In general, useful genes replace or supplement function, including genes encoding missing enzymes such as adenosine deaminase (ADA) which has been used in clinical trials to treat ADA deficiency and cofactors such as insulin and coagulation factor VIII. Genes which affect regulation can also be administered, alone or in combination with a gene supplementing or replacing a specific function. For example, a gene encoding a protein which suppresses expression of a particular protein-encoding gene can be administered. The invention is particularly useful in delivering genes which stimulate the immune response, including genes encoding viral antigens, tumor antigens, cytokines (e.g. tumor necrosis factor) and inducers of cytokines (e.g. endotoxin).

In another aspect the invention provides methods for treating subjects with therapeutics (e.g., cells and tissues) obtained according to the present invention, and for conditions described below and elsewhere herein.

The therapeutics of the invention are administered in effective amounts. The "effective amount" will depend upon the mode of administration, the particular condition being treated and the desired outcome. It will also depend upon, the stage of the condition, the age and physical condition of the subject, the nature of concurrent therapy, if any, and like factors well known to the medical practitioner. For therapeutic applications, it is that amount sufficient to achieve a medically desirable result. In some cases, for example, this is an increase in tissue repair. In other cases, this is an increase in wound healing repair. In further cases, this is an increase in tissue-specific production of a particular protein.

A variety of administration routes are available. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include surgical, oral, rectal, topical, nasal, intradermal, or parenteral routes. The term "parenteral" includes subcutaneous, intravenous, intramuscular, or infusion. A preferred method of administration of cells produced according to any of the methods of the invention is intravenous administration.

In one important aspect the invention involves treatment of subjects with neuronal cells produced according to the invention. Neuronal cells are predominantly categorized based on their local/regional synaptic connections (e.g., local circuit interneurons vs. longrange projection neurons) and receptor sets, and associated second messenger systems. Neuronal cells include both central nervous system (CNS) neurons and peripheral nervous system (PNS) neurons. There are many different neuronal cell types. Examples include, but are not limited to, sensory and sympathetic neurons, cholinergic neurons, dorsal root ganglion neurons, proprioceptive neurons (in the trigeminal mesencephalic nucleus), ciliary ganglion neurons (in the parasympathetic nervous system), etc. A person of ordinary skill in the art will be able to easily identify neuronal cells and distinguish them from non-neuronal cells such as glial cells, typically utilizing cell-morphological characteristics, expression of cell-specific markers, secretion of certain molecules, etc. Differentiating a neuronal cell from another and/or a non-neuronal cell is less important than trying to treat according to the invention the particular region of the nervous system affected (including specific brain regions, e.g., *frontal, parietal, temporal, or occipital lobe*, and spinal cord regions, e.g., *sacral, lumbar, thoracic, or cervical regions*, etc.).

Another important embodiment of the invention, is the treatment of subjects with a neurodegenerative disorder. "Neurodegenerative disorder" is defined herein as a disorder in

which progressive loss of neurons occurs either in the peripheral nervous system or in the central nervous system. Examples of neurodegenerative disorders include: (i) *chronic neurodegenerative diseases* such as familial and sporadic amyotrophic lateral sclerosis (FALS and ALS, respectively), familial and sporadic Parkinson's disease, Huntington's disease, familial and sporadic Alzheimer's disease, multiple sclerosis, olivopontocerebellar atrophy, multiple system atrophy, progressive supranuclear palsy, diffuse Lewy body disease, corticodentatonigral degeneration, progressive familial myoclonic epilepsy, strionigral degeneration, torsion dystonia, familial tremor, Down's Syndrome, Gilles de la Tourette syndrome, Hallervorden-Spatz disease, diabetic peripheral neuropathy, dementia pugilistica, AIDS Dementia, age related dementia, age associated memory impairment, and amyloidosis-related neurodegenerative diseases such as those caused by the prion protein (PrP) which is associated with transmissible spongiform encephalopathy (Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, scrapic, and kuru), and those caused by excess cystatin C accumulation (hereditary cystatin C angiopathy); and (ii) *acute neurodegenerative disorders* such as traumatic brain injury (e.g., surgery-related brain injury), cerebral edema, peripheral nerve damage, spinal cord injury, and Wernicke-Korsakoff's related dementia (alcohol induced dementia). The foregoing examples are not meant to be comprehensive but serve merely as an illustration of the term "neurodegenerative disorder."

Most of the *chronic neurodegenerative diseases* are typified by onset during the middle adult years and lead to rapid degeneration of specific subsets of neurons within the neural system, ultimately resulting in premature death.

In another important aspect, the invention involves treatment of subjects with:

Vascular disorders such as arteriosclerosis, coronary artery disease, aortic aneurysm, arterial diseases of the lower extremities, cerebral vascular disease, congenital or structural vessel defect, inflammatory or granulometers;

Vessel defects, disorders of the smaller vessels; such as hypersensitivity or autoimmune diseases. Other diseases associated with early arteriosclerosis including diabetes mellitus, hypertension, familial hypercholesterolemia, familial combined hyperlipidemia, hypothyroidism, cholesterol storage disease, systemic lupus erythematosis, cystemia, chronic renal insufficiency, chronic vitamin D intoxication, cytoelastocomb itopathic arteriocalsification, aortic valvular calcification.

Diseases of the aorta include aortic aneurysm, as may be caused by artherosclerosis, cystic medial netrosis, siphilitic infection, mycotic infection or rheumatic aortitis or trauma.

Aortic dissection may be caused by cystic medial necrosis, systemic hypertension, arteriosclerosis. Aortic occlusion may be caused by arteriosclerosis, thromboembolism, Giant cell arthritis. Arterial diseases that effect the extremities including arteriosclerosis, fibromuscular dysplasia, thromboengitis obliterans, vasculitis, acute arteriosclerocclusion, artherioembolism, thoracic outlet compression syndrome, arteriovenusphisulas.

Venous disorders of the extremities include venous thrombosis, deep venous thrombosis, superficial veins thrombosis, varicose veins. Examples where epithelial cells may be used, include replacement of urine tract and bladder epiphelium, replacement of skin epithelia, replacement of corneal epithelium, and/or eplacement of intestinal epithelia.

Disorders of the skin include: eczema, psoriasis, papulos sqamis disorders such as: psoriosis, lichenplanus, peteriosis rosia, immunoelectrical mediated skin diseases such as: pamphogus volgarus, pamphogus pholiatious, pamphogoid, dermatitis hapetiformus, epidermal olysis beloza equista, dermatomyocytis, lupus erothemotosis, scleroderma and morphia, burns, ulcers, chromatic lesions, large surgical wounds.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention and are not to be construed to limit the scope of the invention.

Examples

Example 1: *Production of non-hematopoietic lineage cells from hematopoietic progenitor cells*

Experimental Procedures

Culture Devices

The culture devices used in the present invention included: 1) a biocompatible, three dimensional matrix arranged as a series of continuous channels and interconnected pores with a porosity of approximately 90% ("Cellfoam", Cytomatrix, Woburn, MA) coated with full length fibronectin before culture of cells; or 2) multiwell plastic dishes (Corning-Costar, Acton, MA) coated with full length fibronectin (full length fibronectin from Boehringer Mannheim, Germany).

Hematopoietic Stem Cell Isolation

Hematopoietic stem cells obtained from commercially purchased human bone marrow samples (Poietic Technologies, Gaithersburg, MD) were purified by density gradient centrifugation or primary bone marrow preparations over Ficoll 1066 (Sigma, St. Louis, MO). In instances in which $CD34^+$ and $CD34^-$ cells were separated, the Ficoll-purified preparations

were run over a magnetic cell separation column (Miltenyi Biotec, Auburn, CA). To establish the cultures, 66-160x10^5 $CD34^+$ cells were seeded onto the plastic 48-well dishes coated with fibronectin (plastic), or into fibronectin-coated Cellfoam (Cytomatrix) also in 48 well dishes.

Culture Media

Cultures utilized various types of media designed to promote the tissue-specific maturation of the HSCs.

HSC cultures: For HSC cultures, $CD34^+$ or $CD34^-$ cells were cultured in RPMI (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma), penicillin (10 IU/mL), streptomycin (10 μg/mL), L-glutamine (1 mM), Hepes (10 mM), fungizone (2.5 μg/ μL) and the hematopoietic growth factors IL-3 (20 ng/mL), SCF (50 ng/mL) and Flk-2/Flt-3 ligand (50 ng/mL) (all cytokines were from Stem Cell Technologies, Vancouver, CANADA).

Mesenchymal Cell Directed Differentiation: For mesenchymal cell directed differentiation $CD34^-$ cells were cultured in DMEM (low glucose, Life Technologies, Gaithersburg, MD), supplemented with 10% FBS,), penicillin (10 IU/mL), streptomycin (10 μg/mL), L-glutamine (1 mM), Hepes (10 mM), fungizone (2.5 μg/μL) plus the growth factors bFGF (50 ng/mL) (R&D Systems, Minneapolis, MN) and TGF-β (50 pg/mL) (R&D).

Neuronal Cell Directed Differentiation: For neuronal cell directed differentiation cultures, $CD34^+$ cells were cultured in DMEM/F12 medium 1:1 (Life Technologies) containing), penicillin (10 IU/mL), streptomycin (10 μg/mL), L-glutamine (1 mM), Hepes (10 mM), fungizone (2.5 μg/μL), putrescine (9.6 ng/mL), progesterone (6.3 ng/mL), sodium selenite (5.2 ng/mL), insulin (0.025 mg/mL), transferrin (0.1 mg/mL), EGF (20 ng/mL), NGF (20 ng/mL), and bFGF (20 ng/mL) (all from Sigma).

Epithelial Cell Directed Differentiation: For epithelial cell directed differentiation cultures, $CD34^-$ cells were cultured using the same medium formulation as for mesenchymal with the additional supplementation of IL-3 (20 ng/mL), SCF (50 ng/mL), TGF-β1 (50 pg/mL), and Flk-2/Flt-3 ligand (50 ng/mL).

Endothelial Cell Directed Differentiation: For endothelial cell directed differentiation cultures, $CD34^-$ cells were cultured using the same medium formulation as for mesenchymal cells with the additional supplementation of vascular endothelial growth factor (VEGF) (30 ng/mL) (Pharmingen, NJ).

Parenchymal Cell Directed Differentiation: For parenchymal cell directed differentiation cultures, $CD34^-$ cells were cultured using the same medium formulation as for mesenchymal cells with the additional supplementation of EGF, bFGF, and scatter factor (also known as hepatocyte growth factor (SF/HGF).

In all of the foregoing conditions, half media changes were performed twice a week. Cultures were performed in duplicate.

After each incubation period, all cells (adherent and nonadherent) were harvested from all culture conditions. Non-adherent cells were harvested from three dimensional Cellfoam devices by centrifugation for 10 minutes at 1500 rpm (approx. 250 G). Adherent cells from three dimensional culture devices were collected by incubating Cellfoam with Cell Dissociation Solution (Sigma) for 30 minutes at 37°C and then centrifuging at 1500 rpm for 10 minutes. Nonadherent cells from plastic (2D) culture devices coated with fibronectin were collected by gentle washing and adherent cells were collected with CDS by incubation 10 minutes at 37°C. Both adherent and nonadherent cells were pooled and viable cells were counted.

After 2 and 4 weeks of culture, all cells (adherent and non-adherent) were harvested from all of the foregoing culture conditions, counted, and stained with fluorochrome-conjugated antibodies to surface antigens. Antibodies used for surface phenotype determination were chosen based upon the cell type intended to be examined. These included the following:

Hematopoietic cells were identified based on anti-CD34 (Qbend10, Immunotech, Westbrook, Maine), anti-CD38 (Becton Dickinson, San Jose, CA) and-CD45 (Becton Dickinson) monoclonal antibodies to evaluate progenitor cell distributions.

Mesenchymal cells were identified based on vimentin expression using an anti-vimentin monoclonal antibody (Sigma, St. Louis, MO).

Neuronal cells were identified based on expression of neurofilament protein using an anti-neurofilament monoclonal antibody (Sigma, St. Louis, MO).

Epithelial cells were identified based on cytokeratin expression using an anti-cytokeratin antibody (Sigma, St. Louis, MO).

Endothelial cells were identified based on von Willebrand factor expression using an anti-von Willebrand factor monoclonal antibody (Coulter Immunotech, Pittsburgh, PA).

Parenchymal cells can be identified based on a number of different methods well known to those of ordinary skill in the art. For example, kidney cells can be characterized by positive expression of erythropoetin, liver cells can be characterized by positive expression of

albumin, beta-islet cells of the pancreas can be characterized by positive expression of insulin, etc.

Cells were stained in the presence of staining buffer (PBS with 2% fetal bovine serum). After staining, the cells were fixed with 2% paraformaldehyde. Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson). Appropriate controls included matched isotype antibodies to establish positive and negative quadrants, as well as appropriate single color stains to establish compensation. For each sample, at least 20,000 list mode events were collected.

In experiments examining the tissue cross-over of $CD34^-$ cells, $CD34^-$ cells isolated as the eluent from $CD34^+$ purification procedures were cultured in flasks overnight to allow the adherence of cells to the plastic. The following day, the non-adherent $CD34^-$ fraction was isolated and placed into Cellfoam cultures. Cells were harvested at 3 and 6 weeks, and the cells analyzed for functional activity in CFU assays. Interestingly, these cells did not form standard myeloid or erythroid colonies, even when additional cytokines (SCF, IL3 and Flk-2 ligand) were added. Instead, they yielded small, fibroblast-like colonies comprised of small, atypical cells only. Further, in standard LTCIC assays (which includes co-culture with irradiated bone marrow stromal cells followed by plating in methylcellulose), cells from Cellfoam cultures seeded with the $CD34^-$ fraction yielded up to five times as many LTCICs as parallel plastic cultures. These LTCICs, however, were not noticeably different from LTCICs derived from $CD34^+$ cells. This suggests that exposure of the cells from the $CD34^-$ cultures to bone marrow stroma may have primed the cells to become normally responsive to subsequent treatment with cytokines in the CFU assay.

Results

To assess the multi-tissue potentiality of different hematopoietic fractions, $CD34^-lin^-$ cells were isolated from human bone marrow using a magnetic cell separation system (Miltenyi) followed by selection over a column for lineage-positive v. lineage-negative cells. The lineage-negative cells derived from this depletion, whether beginning with $CD34^+$ or $CD34^-$ fractions, were the targeted cells used in the subsequent studies. Following isolation, these cells were then assessed for purity, as were all cells following separation in all experiments described herein, for the expression of CD34, CD45 and a series of lineage positive antigens (CD3, 4, 8, 15, 61, 71, vimentin, cytokeratin, neurofilament and von Willebrand's factor). As determined by these analyses, the cell population yielded from the $CD34^+/^-$ selection process followed by the lineage depletion step was homogeneous, being

typically greater than 97% $CD34^+$ or $CD34^-$ (depending on the approach), >95% CD45% and >99% lineage negative.

To begin the assessment of tissue multipotentiality, we first assessed the ability of $CD34^-$ $CD45^+$ hematopoietic cells to differentiate into $cytokeratin^+$ epithelial cells. 160,000 $CD34^-lin^-$ cells were cultured in fibronectin-coated 48 well plastic culture dishes. After 3 weeks of culture in epithelial-cell specific medium (RPMI media supplemented with 10% FBS plus IL-3 (20ng/ml), SCF (50 ng/ml), FLK-2/FLT-3 ligand (50 ng/ml), bFGF (50ng/ml) and TGF-beta1), adherent and nonadherent were harvested and stained with antibodies against the hematopoietic surface antigen CD45 and the intracellular epithelial cell protein cytokeratin. Cells were gated on CD45. To exclude HPCs, we gated on cells negative or low for CD45 were further analyzed for cytokeratin expression. We observed that in replicate cultures from a homogeneous pool of hematopoietic progenitor cells, a clear population of $CD45^-$ $cytokeratin^+$ epithelial cells was generated under appropriate conditions.

To extend this analysis, $CD34^-lin^-$ cells were also cultured at 140,000 cells per well in the three dimensional device coated with fibronectin (fibronectin-coated Cellfoam). After 7 weeks of culture under the conditions that induce differentiation into vimentin+ mesenchymal cell (DMEM low glucose, 10% FBS, penicillin (10 IU/mL), streptomycin (10 μg/mL), L-glutamine (1 mM), Hepes (10 mM), fungizone (2.5 μg/μL) plus the growth factors bFGF (50 ng/mL) and TGF-beta (50 pg/mL), cells were harvested and adherent and nonadherent cells were pooled together. Cells were then seeded at 10,000 cells per plate in methylcellulose to examine their colony forming potential following a period in semi-solid medium. After 19 days, colonies were isolated from the methylcellulose and, after overnight incubation in RPMI medium, were stained for CD45 and the intracellular mesenchymal cell protein vimentin. Cells were gated on CD45 and cells negative for CD45 (to exclude HPCs) were further analyzed for vimentin expression. We observed that under these conditions, a pure population of hematopoietic cells is able to form $CD45^-$ $vimentin^+$ mesenchymal cells.

In a separate series of studies examining the multi-tissue potential of $CD34^+$ cells, $CD34^+lin^-$ cells were isolated from human bone marrow and 100,000 cells per well were cultured in the three dimensional matrix Cellfoam under conditions that induce the formation of $neurofilament^+$ neural cells (DMEM/F-12 media supplemented with 10% FBS, progesterone (6.3 ng/ml), putrescine (9.6 ng/ml), sodium selenite (5.2 ng/ml), insulin (0.025 mg/ml) and transferrin (0.10 ng/ml)). After 4 weeks of culture, cells were harvested and stained with antibodies against CD45 and the intracellular neuronal cell neurofilament

protein. After gating on CD45 negative cells (to exclude HPCs), the cells were analyzed for neuronal cells containing neurofilament. We observed from the data obtained a population of non-hematopoietic, neurofilament$^+$ neuronal-like cells that were produced from the CD34$^+$ hematopoietic cell sample.

To further assess the production of tissue cross-over cells from hematopoietic progenitors, standard cytomorphological studies were carried out. In these studies, cells from the various cultures conditions, including those described above, were resuspended at 1,000 cell per μl in PBS. 50 μl of this suspension was then affixed to a glass microscope slide by centrifugation in a cytocentrifuge at 600 rpm for 30 seconds. The slides with affixed cells were then removed, air dried and stained with a standard hematoxylin/eosin staining. The slides were then scored by qualified technicians for the presence of the targeted tissue-specific cell types. In each case, the culture of CD34$^+$ or CD34$^-$ hematopoietic cells (depending on the condition) in media that induced the differentiation of the specified non-hematopoietic cell types led to the detectable presence of cells with the typical morphology of the targeted cell type. For example, culture of CD34$^-$ cells under mesenchymal cell conditions led to the formation of a large number of cells with mesenchymal-like appearance that had large, fusiform bodies with centrally placed, round nuclei and tapered cytoplasmic processes at their ends. Conversely, culture of CD34$^+$ cells under neuronal cell conditions led to the formation of cells that resembled neuronal cells having extremely long cell bodies, very thin cell bodies and extensive cytoplasmic processes. This work corroborated at the qualitative level the quantitative data generated by the flow cytometric analyses.

Further, directly comparing the production of lineage-targeted, non-hematopoietic cells from either the CD34$^+$ or CD34$^-$ fractions, comparison of the culture and differentiation processes in the three dimensional matrix device (Cellfoam) to the results obtained in standard plastic monolayer controls revealed that the yields of non-hematopoietic lineage-specific (lin$^+$) cells was often superior in Cellfoam. For example, when CD34$^-$ cells from primary bone marrow samples were cultured in the mesenchymal cell-specific medium (DMEM base supplemented with mesenchymal specific cytokines as noted above), the three dimensional cultures routinely yielded more CD45$^-$ (non-hematopoietic) vimentin+ (mesenchymal) cells than plastic cultures. For example, as shown in Figure 1, Cellfoam cultures produced between 3.5 and 12 times as many cells with this lineage-specific phenotype as plastic cultures did in 5 out of 5 replicates (conditions A-E). Similarly, when CD34$^+$ cells from primary bone marrow samples were cultured in neuronal cell-specific

medium (DMEM/F12 base supplemented with neuronal specific cytokines as noted above), the three dimensional matrix cultures produced significantly more $CD45^-$ (non-hematopoietic) $neurofilament^+$ (neuronal) cells than plastic cultures. As shown in Figure 2, the three dimensional matrix cultures yielded up to 160 fold more cells in multiple replicates as plastic under the neuronal-cell specific culture conditions (DMEM/F12). However, in the absence of neuronal cell specific conditions (R-10+HSC, Figure 2), there was no difference between the three dimensional and monolayer control cultures.

Example 2: *Production of non-hematopoietic lineage cells from $CD34^-Lin^-$ hematopoietic progenitor cells and in vivo homing of the non-hematopoietic lineage cells to a tissue of interest*

Cell Culture

The culture devices used in these studies consisted of the following:

1) Cellfoam (as described above -Cytomatrix, Woburn, MA) coated with full-length fibronectin (Boehringer Mannheim, Germany) prior to adding cells; and 2) multiwell plastic dishes (Corning-Costar, Acton, MA) coated with full-length fibronextin (Boehringer Mannheim, Germany).

$CD34^-Lin^-$ Cell Isolation

Normal human bone marrow cells or light density mononuclear cells were purchased from Poietic Technologies (Gaithersburg, MD). In case of whole bone marrow cells mononuclear cells were isolated by diluting the samples 1:3 with RPMI-1640 media followed by Ficoll-hispaque density (1.077 mg/cm^3) centrifugation separation (Sigma). Light density mononuclear cells were resuspended in phosphate-buffered saline (PBS) without Ca^+, Mg^+ supplemented with 2% FBS and incubated at room temperature for 15 minutes with a mixture of CD2, CD3, CD11b, CD14, CD15, CD16, CD19 CD24, CD33, CD41, CD56, CD66b, and Glycophorin-A tetrameric antibody complexes (StemCell Technologies, Vancouver, British Columbia, Canada). These tetrameric complexes are bispecific cross-linkers that bind that described antigen and dextran. After washing excess antibodies, 20 nm magnetic colloidal iron/dextran particles were added and incubated at room temperature for 15 minutes. Cells wre then eluted through a magnetic column to enrich for cells not expressing lineage markers (Lin^-) according to the manufacture's instructions (STEMSEP™, StemCell Technologies). Lineage depleted cells were subjected to a standard CD34 immunomagnetic bead separation using the miniMACS system following the manufactuerer's guidelines (Miltenyi Biotech, Auburn, CA). Both $CD34^+Lin^-$ and $CD34^-Lin^-$ population of cells were collected.

In Vitro Long-term cultures

To establish the long-term cultures, 100-120x10^3 $CD34^+Lin^-$ and $CD34^-Lin^-$ cells were seeded onto the plastic 48-well dishes coated with fibronectin (plastic), or into fibronectin coated Cellfoam (Cytomatrix, Woburn, MA) also in 48-well dishes. Cultures utilized various types of media designed to promote different cell types differentiation. For hematopoietic stem cell, 1 ml of RPMI (Sigma, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (Sigma, St. Louis, MO), Penicillin (10 IU/ML), Streptomycin (10 μg/ml), L-glutamine (1mM), Hepes (10mM), Fungizone 2.5 μg/ml (Life Technologies, Gaithersburg, MD). The following human recombinant cytokines were added IL-3 at 20 ng/ml, SCF and FLK-2 ligand both at 50 ng/ml (StemCell Technologies, Vancouver, British Columbia, Canada). For mesenchymal stem cells, cells were cultured in DMEM containing low glucose (Life Technologies, Gaithersburg, MD) supplemented with 10% Fetal Bovine Serum (Sigma), Penicillin (10 IU/ML), Streptomycin (10 μg/ml), L-glutamine (1mM), Hepes (10mM), Fungizone 2.5 μg/ml (Life Technologies, Gaithersburg, MD) and 100nM Dexamethasone, 0.05 mM L-Ascorbic acid, 10mM β-Glycerophosphate and bFGF at 50 ng/ml (R&D system, Minneapolis, MN), TGF-β at 50 pg/ml (R&D system, Minneapolis, MN), EGF at 25 ng/ml and VEGF at 10 ng/ml. (Pharmingen, San Diego, CA). For neuronal stem cells, DMEM/F-12 media (Life Technologies, Gaithersburg, MD) supplemented with 10% Fetal Bovine Serum (Sigma), Penicillin (10 IU/ML), Streptomycin (10 μg/ml), L-glutamine (1mM), Hepes (10mM), Fungizone 2.5 μg/ml (Life Technologies, Gaithersburg, MD), Putrescine at 9.6 ng/mg (Sigma, St. Louis, MO), Progesterone at 6.3 ng/ml (Sigma, St. Louis, MO), Sodium Selenite at 5.2 ng/ml (Sigma, St. Louis, MO), Insulin at 0.025 mg/ml (Sigma, St. Louis, MO), Transferrin at 0.1 mg/ml (Sigma, St. Louis, MO) and bFGF at 50 ng/ml (R&D system, Minneapolis, MN) EGF at 25 ng/ml and β-nerve growth factor at 100 ng/ml (R&D system, Minneapolis, MN) concentrations were used. For all cultures, half media changes were performed twice a week. Cultures were performed in duplicate.

After each incubation period, all cells (adherent and nonadherent) were harvested from all culture conditions. Non-adherent cells were harvested from three-dimensional Cellfoam devices by centrifugation for 10 minutes at 1500 rpm (approx. 250 G). Adherent cells from three dimensional culture devices were collected by incubating cellfoam with Cell Dissociation Solution (Sigma, St. Louis, MO) for 30 minutes at 37°C or treated with trypsin for 10 minutes at 37°C and terminating the reaction with FCS and then centrifuging at 1500 rpm for 10 minutes. Nonadherent cells from plastic coated with fibronectin cultures were

collected by gentle washing and adherent cells were collected with CDS by incubation 10 minutes at 37°C. Both adherent and nonadherent cells were pooled and viable cells were counted.

Transplantation

Human cell preparation *in vitro* cultures: 500,000 $CD34^{+}Lin^{-}$ cells were seeded into 6 well fibronectin coated dishes. Cells were cultured for 4 weeks in neuronal growth medium and growth factors with half of medium change biweekly. After harvest as described above, adherent and nonadherent cells were pooled and resuspended in PBS as 350,000/0.300 ml.

Mice: NOD/SCID mice 4-6 weeks old were purchased from Jackson Laboratories (Maine). Mice were irradiated with 325 cGy of total body irradiation from $coblt^{60}$ source. After 24 hours of radiation, mice were injected with above mentioned human cells or PBS as control. All mice were handled under sterile condition and maintained in cage microisolators. Mice were sacrificed 18 days postransplantation by cervical dislocation. Brain was isolated immediately followed by thymus, spleen, bone marrow, and muscle.

Cell preparation from different tissues:

i) Preparation of Brain cells: All the isolation process was performed using an ice cold sterile PBS, GKN buffers made by adding 8 g/L NaCl, 0.4g/L KCl, 3.56 g/L $NaHPO_4$. $12H_2O$, 0.78 g/L $NaH_2PO4.2H_2O$, 2g/L and D-(+) glucose, pH 7.4. Before isolation 0.02% (w/v) isotonic BSA was added into GKN buffer. Buffer for enzymes were made by adding 4g/L $MgCl_2$, 2.55 g/L $_{cacl1}$, 3.73g/L KC1, 8.95 g/L NaCl in PBS, pH=6-7. Mice brains were isolated within minimum time and kept in ice-cold GKN buffer+0.02% BAS and divided into two pieces. Half of the brain was fixed with formalin for IHC. The other half was used to isolate cells for FACS and PCR. Mechanically the brain was dissociated into pieces with forcepts and passed through 70 μm filter unit and collected in a 50 ml tube and washed by centrifugation for 10 min at 400g. The pellet was digested emzymatically with collagenase and Dnase for 60 min at 37°C. During the digestion the cells were resuspended with pipette tips. After digestion the cells were washed twice with 40 ml of GKN/BSA buffer. The cells were resuspended in 10 ml of Percoll 1.03 g/ml and underlayered with 5 ml of 1.095 g/ml of Percoll and overlayed with 5 ml of GKN/BSA buffer. Centrifugation was done for 30 min at 20°C at 1250Xg with break off position. Cells were collected from the top of 1.095 g/ml Percoll and washed with 50 ml of GKN/BSA buffer by centrifugation for 10 min at 400g. Cell pellets were resuspended in 2 ml GKN/BSA buffer and layer over second gradient in 10 ml tube which consisted of 1.088 g/ml, 1.072 g/ml, 1.03 g/ml Percoll. A second density

centrifugation was done at 1250g for 20 min at 20°C break off position. Cells from 1.071 g/ml and 1.088 g/ml interfaces were collected and washed with 50 mg of GKN/BSA buffer.

ii) Preparation of Muscle, Thymus, Spleen cells:

Muscle Tissues were divided into two. Half was fixed with 36% formalin for IHC and other half was used to isolate the cells. Half of the muscle tissues, thymus, and spleen were minced with stainless scissors separately and grinned. Spleen and thymus were filtered through 70 μm filter unit and collected separately. The muscle cell pellet was treated with 0.2% Collagenase at 45°C for 45 minutes. After inhibiting trypsin with FBS, cells were washed and passed through 70 μm filter unit.

iii) Preparation of bone marrow cells:

Femurs, tibiae and glumerules of each mouse were collected. Bone marrow cells were obtained by flushing the shafts of the bones with 2% FBS containing PBS using 1 ml syringe and 21 gauge and 27 gauge needles.

Flow cytometry analysis

After 3 to 6 weeks of culture, all cells (adherent and non-adherent) were harvested from all culture conditions, counted, and stained with fluorochrome-conjugated antibodies to surface antigens and in some cases for intracellular filamentous proteins. Antibodies used for surface phenotype determination included anti-CD34 (Qbend10, Immunotech, Westbrook, Maine), anti-CD38 (Becton Dickinson, San Jose, CA) and-CD45 (Becton Dickinson) antibodies to evaluate progenitor cell distributions. Cells were stained in the presence of staining buffer (PBS with 2% fetal bovine serum). After staining, the cells were fixed with 2% paraformaldehyde. For intracellular proteins, Fix and Perm kit from Caltag Laboratories (Burlingame, CA) and antibodies to neurofilament (DAKO, Carpintera, CA), Von Willebrand's factor (Coulter-Immunotech, Miami, Florida), Cytokeratin and Vimentin (Sigma, St. Louis, MO) were used following the stained protocol of the manufacturer.

To detect human cells in transplanted mice tissues, cells from multiple tissues were resuspended at $1x10^6$ cells in 0.100 ml of 2% FBS containing PBS buffer concentration. To block nonspecific binding cells were then incubated 30 minutes at 4°C with antibody to murine Fc receptor. Cells were then stained with antibodies to human CD45, human MHC class-1 molecule for surface staining and with antibody vimentin for intracellular staining. Aliquots of every tissue sample were stained isotype control antibodies conjugated to PE, FITC and APC. To examine any cross reactivity between antibodies to mouse CD45 (FITC-conjugate) with human CD45 (APC-conjugate), aliquots of each tissue sample were also stained with these two antibodies.

Flow cytometry was performed using a FCSCalibur flow cytometer (Becton Dickinson). Appropriate controls include matched isotype antibodies to establish positive and negative quadrants; as well as appropriate single color stains to establish compensation. For each sample, at least 20,000 list mode events were collected.

Colony-formation assays

To assess the colony forming ability of cells isolated from the cultures in Cellfoam or plastic, traditional methylcellulose assays were performed. For hematopoietic colony assays, equal numbers of cells isolated from Cellfoam or plastic/FN coated cultures, as described above, were added to $6.67x10^4$ cells/ml to 3.0 ml of methylcellulose medium with cytokines (IL-3 20 ng/ml: erythropoietin 3 IU/ml; stem cell factor 50 ng/ml; all Stem Cell Technologies, Vancouver) plus 0.5 ml of DMEM (2% FCS, 10 IU/ml penicillin, 10 μg/ml streptomycin, 1 MM L-glutamine). 1.5 ml of this mixture was added to a 35 mm graduated petri dish. For nonhematopoietic colony assays, equal numbers of cells isolated from Cellfoam or plastic/FN coated cultures 25,000 were added to the 3.0 ml of methylcellulose medium with bFGF, TFG-β and EGF. Duplicate assays were performed for each condition. The duplicate petri dishes were then placed in an incubator with 5% CO2 containing humidified atmosphere at 37°C for 10-21 days. Following the incubation period, the number of colonies was determined by light microscopy. Positive colonies were scored on the basis of an accumulation of 40 or more cells. Counts were done in duplicate.

PCR amplification of globin DNA

Genomic DNA was prepared from cells using the QIAamp DNA Mini Kit (Qiagen, Santa Clarita, CA). Human-specific primer sequences were: 5'-GGGCAAGGTGAAGG TGAACGTGGATGA-3' (SEQ ID NO:1) and 5'-CCATCACTAAAGGCACCGAGC-3' (SEQ ID NO:2) and 5'-GTCACCAGCAGGCAGTGGCT-3' (SEQ ID NO:3). All primers were synthesized by GIBCO/BRL.

PCR conditions were: 94°C for 2 min, followed by 95°C, 30 s, 58°C 30 s and 72°C, 45 s for 38 cycles. One second per cycle was added to the elongation time for the final 28 cycles. Each PCR reaction contained 2 U of Expand polymerase mix (Boehringer Mannheim, Germany), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 12.5 μM each primer.

Results

To investigate the multi-potentiality in differentiation of human bone marrow derived $CD34^-Lin^-$cells, we performed experiments examining the effects of a series of different,

targeted culture conditions. $CD34^-Lin^-$ cells isolated from human adult bone marrow were used in cultures specific for hematopoietic stem cell (HSC), mesenchymal stem cell (MSC) and neural stem cell (NSC) differentiation. $CD34^+Lin^-$ cells were also collected and treated in same was as $CD34^-Lin^-$ cells. After 3 to 4 weeks, the cultures were harvested and evaluated by flow cytometry. MSC cultures, which consisted of MSC growth medium and growth factors, resulted in the development $CD45^-Cytokeratin^+$ (epithelial) and $CD45^-Vimetin^+$ (mesesnchymal) cells with $CD34^-lin^-$ cell. When vascular endothelial growth factor was added to the culture medium, cells expressing von Willebrands Factor were detected in the MSC cultures as indicated by Figure 3B (left panel). $CD34^+Lin^-$ cells did not express any epithelial cell or endothelial cells surface markers. Non-adherent cells expressing the hematopoietic phenotype $CD45^+CD34^+CD38^+$ were isolated from hematopoietic growth culture medium supplemented with hematopoietic growth factors using both $CD34^-lin^-$ and $CD34^+Lin^-$ cells. We observed that only $CD34^-Lin^-$ cells cultured in neuronal growth medium supplemented with neuronal growth factors were observed to express neurofilament (Figure 3C). The morphology of the cultured $CD34^-lin^-$ cells in these three growth specific culture media was also markedly different. Long adherent cells were extensive complex process as were seen in neuronoal cultures. Adherent, spindle shaped cells were observed in MSC cultures. Round nonadherent were observed in hematopoietic cultures.

In summary, the data indicate that bone marrow derived $CD34^-lin^-$ cells appear to contain a primitive precursor cell or pool of cells which can be manipulated *ex vivo* to differentiate into a particular non hematopoietic tissue type.

To extend this work and assess the *in vivo* attributes of *in vitro*-derived cells, bone marrow $CD34^-Lin^-$ cells cultured for 4 weeks under conditions for neuronal cell differentiation were injected via the tail vein into irradiated NOD/SCID mice. After 18 days, the mice were sacrificed and cells from the brain, muscle, thymus, spleen, and bone marrow were collected and stained with antibodies specific to human CD45 and MHC Class-1. Figure 4 shows the presence of human cells ($CD45^+$ and/or MHC Class 1^+) in the brain of transplanted mice. Although, under normal physiological conditions, the blood brain barrier prevents cell migration into the brain, it has been reported that during transplantation of hematopoietic cells, a small population from donor immature cells enters the brain (Ono K., et al., *Biochem. & Biophys. Res. Comm*, 1999, 262:610-614). Homing of the injected cells only to the brain indicates the organ-specific homing of the transplanted cells. To confirm this flow cytometry observation, PCR specific for human globin was performed with brain and muscle cells of the transplanted and nontransplanted mice. The PCR results as shown in

Figure 5 demonstrate the presence of human cells only in the brain of the transplanted mice. Further analyses of cells isolated from the bone marrow, thymus and spleen of the transplanted mice indicated the absence of human cells from any of the foregoing tissues.

These data indicate that HSC, MSC and NSC may originate from a common precursor or pool of precursors in human bone marrow. These precursor cells may have strong requirements for defined growth environments to enable their differentiation into particular lineages. The data also suggests that the homing of the injected cells is organ specific.

All references disclosed herein are incorporated by reference in their entirety.

We claim: